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The prognostic value of fast molecular response of marrow disease in patients aged over 1 year with stage 4 neuroblastoma

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ABSTRACT

Background: Quantitative real-time (q)PCR for detection of minimal residual disease (MRD) in children with neuroblastoma (NB) can evaluate molecular bone marrow (BM) response to therapy, but the prognostic value of tumour kinetics in the BM during induction treatment remains to be established. The purpose of this study was to analyse at which time points MRD detection by sequential molecular assessment of BM was prognostic for overall survival (OS).

Methods: In this single centre study, qPCR was performed with five NB-specific markers: *PHOX2B*, *TH*, *DDC*, *GAP43* and *CHRNA3*, on 106 retrospectively analysed BM samples of 53 patients >1 year with stage 4 neuroblastoma. The prognostic impact of MRD at diagnosis ($n = 39$), at 3 months after diagnosis ($n = 38$) and after completing induction chemotherapy ($n = 29$) was assessed using univariate and bivariate Cox regression analyses.

Results: There was no correlation between tumour load at diagnosis and outcome ($p = 0.93$). Molecular BM remission was observed in 11/38 (29%) of patients at 3 months after diagnosis and associated with favourable outcome (5-y-OS 62 ± 15.0% versus 19 ± 8%; $p = 0.009$). After completion of induction chemotherapy, BM of 41% (12/29) of the patients was still MRD positive, which was associated with poor outcome (5-y-OS 0% versus 52 ± 12%; $p < 0.001$). For both time points, the prognostic value of molecular response remained significant in bivariate analysis.

Conclusions: MRD detection measured by a panel of NB specific-PCR targets could identify fast responders, who clear their BM early during treatment. Fast molecular response was a prognostic factor, associated with better outcome. Our data indicate that MRD analysis during induction therapy should be included in prospective MRD studies.

1. Introduction

Neuroblastoma (NB) is the most common extra-cranial solid neoplasm in children. More than 50% of the patients present with metastasis at the time of diagnosis, with a 5-year survival rate of only 30–40%.[1] Especially bone marrow (BM) involvement is very common in children with metastatic disease detected by standard morphologic examination. Detection of tumour cells by quantitative real-time (q)PCR with a panel of neuroblastoma specific RNA markers was shown to

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be more sensitive than conventional cytology and this technique reliably detects tumour cells with a sensitivity of 1 in 10^6 normal nucleated bone marrow cells.[2,3] Currently, overall response evaluation is based on the International Neuroblastoma Response Criteria (INRC),[4] in which results on BM cytology, MIBG scintigraphy, urinary catecholamine excretion and imaging (echo/MRI) are combined to assess the extent of disease. However, detection of minimal residual disease (MRD) in BM and/or peripheral blood (PB) using qPCR could be more significant for therapy response evaluation. Sensitive MRD measures for early response to therapy might predict ultimate response and survival. Identification of patients with resistant tumours shortly after the start of therapy would enable a choice for alternative treatments and might improve survival rates.

Although MRD using qPCR is widely studied in neuroblastoma, still little information is available on the correlation of MRD at different time points during treatment with clinical outcome. Evaluation of tumour cell content in BM during therapy using immunocytology has shown to provide important prognostic information in metastatic NB.[5] In addition, there is increasing evidence that also MRD PCR markers can be useful for the evaluation of therapeutic response.[6–8]

The purpose of this study was to determine whether sequential molecular MRD assessment of bone marrow during the first year of therapy was prognostic for ultimate overall survival (OS). We report here the correlation between outcome and bone marrow MRD status measured by a panel of NB-specific PCR targets, for all assessable BM samples of patients with metastatic high risk neuroblastoma treated between 1991 and 2006 in the Emma's Children Hospital in the Netherlands.

2. Material and methods

2.1. Patients and samples

In this retrospective study we investigated archived BM samples from children older than 1 year of age, diagnosed with stage 4 neuroblastoma staged according to the International Neuroblastoma Staging System (INSS),[4] treated at the Emma Children's Hospital/AMC (Amsterdam, The Netherlands) between 1991 and 2006. In total, 106 BM samples were collected from 53 patients at the following time points; 39 at diagnosis; 38 at mid induction (3 months after diagnosis; range 2–4 months) and 29 at end induction (median 8 months after diagnosis, range 6–12 months), see Table 1. We have previously extensively shown that all BM samples positive with cytology at diagnosis are also positive by qPCR,[2,9] so for the analyses at mid- and end-induction patients with BM disease detected by cytology and/or qPCR at diagnosis were included. Data extracted from the medical record included dates of diagnosis and death, MYCN gene copy number, results of bone marrow aspirates and biopsies, ^{123}I-meta-iodobenzylguanidine (^{123}I-MIBG) scan, ultrasound, CT scan or MRI scan, and urine catecholamine metabolites (homovanillic acid (HVA) and 4-hydroxy-3-methoxymandelic acid (HMM), formerly named vanilmandelic acid (VMA). Disease status at mid induction and end induction was scored according to the International Neuroblastoma Response Criteria (INRC).[4] Written informed consent was obtained from parents or guardians. The study was approved by the Medical Research Ethics Committee of the AMC.

2.2. Therapy

Patients were treated according to consecutive protocols used at the Emma Children's Hospital/AMC, Amsterdam, in the Netherlands; these protocols used the following induction chemotherapy: VECI[10] ($n = 36$), rapid-COJEC[11] ($n = 7$) or GPOH-NB97[12] ($n = 10$). Prior to induction chemotherapy, a majority of patients received (1–4) infusions of ^{131}I-MIBG ($n = 36$) as described before.[10] In 7 patients infusions of ^{131}I-MIBG were directly followed by Topotecan ($n = 7$). In all protocols patients were treated with myeloablative chemotherapy (MAT) and re-infusion of autologous bone marrow ($n = 16$) or peripheral blood stem cells ($n = 17$), if they had CR or VGPR, followed by 13-*cis*-retinoic acid therapy.[1]

2.3. RNA extraction, reverse transcription and quantitative real-time PCR

Cells were isolated by haemolysis (NH_4Cl) within 24 h after collection in EDTA tubes. Cells were counted and aliquoted per $5 * 10^6$ in 0.5 ml RNABee. Total cellular RNA was extracted with RNABee method according to manufacturer's instructions (Campro Scientific, Veenendaal, the Netherlands). RNA concentration and quality were determined using an ND-1000 spectrophotometer (Nanodrop, Wilmington, USA). cDNA was synthesised as described previously.[2]qPCR for paired-like homeobox2b (*PHOX2B*), tyrosine hydroxylase (*TH*), dopamine decarbohydroxylase (*DDC*), cholinergic receptor3 (*CHRNA3*) and growth associated protein 43 (*GAP43*) was performed in an ABI PRISM 7900 (PE Biosystems, Darmstadt, Germany) as described before.[2,9] Reference gene beta-glucuronidase (*GUS*) was used for normalisation (normalised Ct (ΔCt) = Ct *GUS* – Ct marker), because it was stably expressed in all NB tumours tested (data not shown) and because its expression in NB was equal to that in haematologic cells.[13] The number of *GUS*-copies was determined using *GUS*-plasmid DNA (Ipsogen, Marseille, France) dilutions. All qPCR experiments were carried out at least in duplicate and mean values were used. An IMR32 calibration curve was used as an exogenous positive control to ascertain the efficiency of each PCR reaction.

2.4. Data analysis

Marker positivity has been described previously.[2] In short, clinical samples were scored positive if *PHOX2B* $Ct_{sample} < 50$ or if one of the other markers had a mean $\Delta CT_{sample} > 3.0$ Ct of $\Delta Ct_{control\ BM}$. Thus if one of the markers was scored positive the samples were scored as positive. Thresholds for positivity ($\Delta Ct_{control\ BM}$) were determined in BM samples from 51 children in molecular remission of acute lymphoblastic leukaemia[9] and depicted in supplementary Table 1.

2.5. Statistical analysis

Correlation analysis was done using scatterplot and Spearman's test. Overall survival was calculated using the

Table 1 – Sample origin.

Patient nr.	Treatment protocol	Diagnosis	Mid induction	End induction	5-year OS (months)	Died
60	VECI		x	x	60	0
64	VECI	x		x	13	1
65	VECI			x	8	1
81[a]	VECI	x			60	0
82	VECI			x	19	1
93	VECI	x			4	1
166	VECI	x	x		60	0
168	VECI			x	14	1
248	VECI		x		5	1
249	VECI		x		4	1
341[a]	VECI	x			41	1
406	VECI	x	x		8	1
407	VECI			x	60	0
410	VECI	x	x		60	0
415	VECI	x	x	x	12	1
418	VECI	x	x		60	0
425	VECI	x		x	41	1
430	VECI	x	x	x	10	1
442	VECI		x	x	12	1
458	VECI	x	x		7	1
462	VECI	x		x	60	0
479	VECI	x	x		16	1
483[a]	VECI	x			60	0
489	VECI	x	x	x	7	1
490	VECI	x	x	x	18	1
492	VECI		x	x	19	1
511	VECI		x	x	60	0
519	VECI		x	x	7	1
538	VECI	x	x	x	11	1
539	VECI		x		47	1
540	VECI	x	x	x	9	1
557	VECI	x	x		12	1
559	VECI	x	x		11	1
576	VECI	x	x	x	46	1
583	VECI	x	x		6	1
593	VECI	x	x	x	60	0
594	Rapid-COJEC	x	x	x	60	0
597	Rapid-COJEC			x	25	1
599	Rapid-COJEC	x		x	9	1
600	Rapid-COJEC	x	x	x	14	1
604	Rapid-COJEC	x	x		20	1
620	Rapid-COJEC	x		x	58	0
621	Rapid-COJEC	x	x	x	56	0
622	GPOH-NB97		x		19	1
627	GPOH-NB97	x	x		10	1
628	GPOH-NB97	x	x		6	1
630	GPOH-NB97	x	x		54	0
635[a]	GPOH-NB97	x			50	0
638	GPOH-NB97	x	x		9	1
642	GPOH-NB97	x	x	x	29	1
649	GPOH-NB97	x	x	x	41	0
652	GPOH-NB97	x	x		41	0
661	GPOH-NB97	x	x	x	19	1
Total sample		39	38	29		

Abbreviations: VECI, vincristine, ifosfamide, carboplatin, etoposide; OS, overall survival in months; died, 0, alive; 1, died.

[a] Patient with RQ-PCR negative BM at diagnosis. These patient's samples were not included in further analyses regarding therapy response of BM disease.

Kaplan–Meier method and survival curves were compared using log-rank statistics. Due to small patient numbers, bivariate Cox models were used to assess the independence of predictive information coming from BM qPCR relative to other prognostic factors and response indicators. Only factors, which were significant in univariate analyses, were assessed in bivariate analyses. All statistics were done using SPSS 15.0.

3. Results

3.1. Patients' characteristics

The median age at initial diagnosis of all 53 stage 4 neuroblastoma patients was 2.5 years (range 1.0– 3.5 years), and the median observation time was 9.6 years (range 3–21 years) (Table 2). At diagnosis all patients had BM or distant bone metastases determined by BM cytology ($n = 46$), MIBG scintigraphy ($n = 6$) and/or bone scintigraphy ($n = 1$). The 5 year overall survival was 33.4 ± 6.6%. No difference in outcome was found between patients treated with the different protocols for induction chemotherapy ($p = 0.60$), patients treated with or without MIBG infusions ($p = 0.37$) and patients reinfused with autologous BM or PBSC grafts ($p = 0.50$). Furthermore, MYCN status, positivity of urine catecholamine metabolites, MIBG and/or BM cytology at diagnosis had no significant bearing on outcome, see for p-values Table 2.

Table 2 – Characteristics of stage 4 neuroblastoma patients >1 year of age ($n = 53$).

Characteristic		p-Value[a]
Age at diagnosis		0.19
Median (months)	30	
Range	12–162	
<18 months of age	8	
>18 months of age	45	
MYCN		0.07
Amplified	18	
Not amplified	35	
BM cytology		0.30
Positive	46	
Negative	7	
Urinary catecholamines		0.06
Positive	50	
Negative	3	
MIBG scan initial diagnosis		
MIBG uptake by primary tumour		0.17
Positive	49	
Negative	4	
MIBG uptake by BM/bone metastasis		0.32
Positive	44	
Negative	9	
BM qPCR		0.10
Positive	35	
Negative	4	
Missing	14	
Treatment		
MIBG therapy		0.37
MIBG upfront	36	
MIBG + topotecan upfront	7	
No MIBG upfront	10	
Protocol		0.60
VECI	36	
rCOJEC	7	
GPOH-NB97	10	
Autologous stem cell transplantation		0.50
BM	19	
No reinfusion[b]	3	
PBSC	23	
No reinfusion[b]	6	
No harvest[b]	11	
Overall survival after 5 years		
Median percentage	33.4	
Std error	6.6	
Observation time		
Median (months)	116	
Range	36–256	

[a] Log rank test.
[b] No harvest or reinfusion of graft due to progressive disease.

3.2. Results of quantitative real-time PCR

Of the 106 archived BM samples from 53 stage 4 patients; 39 samples collected at diagnosis were available; 38 at mid induction and 29 at end induction. Of this series of 106 BM samples, 74 tested positive by molecular analyses. *PHOX2B* was the most sensitive marker and was detected in 72 of the 74 positive samples, see Fig. 1.

At diagnosis 4 of the samples from 39 patients were negative with all qPCR markers. These patients also had negative BM cytology and were not included in further analyses regarding therapy response of BM disease. *PHOX2B* was the only marker that was positive in all 35 positive samples (data not shown).

In BM at 3 months (range 2–4) after start of therapy, 27 of the 38 samples tested positive for MRD. Of these 27 positive samples, 26 were at least positive for *PHOX2B*, while one sample was only positive for *TH*.

In BM after completion of induction chemotherapy, 12 of the 29 samples were MRD positive. One sample was only positive for *CHRNA3*, while 11 samples were at least positive for *PHOX2B*.

3.3. Outcome according to tumour load in BM at diagnosis

Fig. 2 shows that tumour load at diagnosis, as defined by the normalised expression of *PHOX2B* (Ct *GUS* – Ct *PHOX2B*) in BM, did not correlate to survival ($p = 0.93$). This was also found for the other markers (data not shown). Of the 4 patients without BM involvement at diagnosis measured with qPCR, 3 patients are still alive with a median follow up of 80 months. The other patient died of progressive disease 52 months after diagnosis, see for survival curves Fig. 3a.

3.4. Outcome according to MRD status 3 months after diagnosis

To identify fast molecular responders, we analysed MRD status 3 months (range 2–4) after start of therapy of patients with BM positive disease at diagnosis. Twenty-seven of the 38 patients (71%) had MRD positive BM, so 11 patients (29%) did not have detectable MRD in the BM anymore. The patients with a fast molecular response had significantly better survival than patients, who still had MRD positive BM at that time (5-y-OS of 62.3 ± 15.0% versus 18.5 ± 7.5%, log rank test $p = 0.009$, see Fig. 3b). Partial response (PR) was also associated with better outcome compared to a mixed response (MR) or no response (NR), in univariate analysis. The other clinical

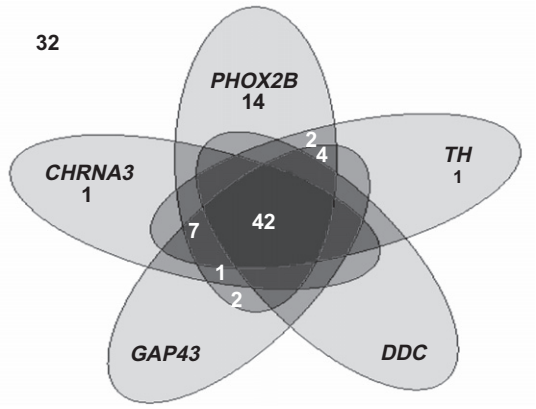

Fig. 1 – Value of markers in MRD detection. A total 106 BM samples were tested with a panel of five qPCR markers. Each ellipse represents positive results of one marker. The number outside the ellipses represents the samples which were negative for all five markers ($n = 32$).

response parameters at this time point, BM cytology, urinary catecholamine response and presence of residual MIBG positive metastasis, were not significantly associated with outcome. In bivariate analysis with INRC response, molecular response remained significantly associated with survival ($p = 0.05$) (Table 3), indicating that qPCR can give information, which cannot be obtained with the currently used response parameters in predicting survival. Fifteen of 27 MRD positive patients never reached VGPR and progressed before autologous harvest or stem cell reinfusion could be performed.

Results of individual markers were not prognostic for outcome, except for PHOX2B (5-y-OS of 57.3 ± 14.6% versus 19.2 ± 7.7%, log rank test $p = 0.011$). However, with the use of a panel of markers, one more positive sample was identified, therefore, the panel was more prognostic for outcome ($p = 0.009$).

Of the whole cohort of 53 patients there were 29 that could be analysed with five markers both at diagnosis and at mid-induction. The levels at diagnosis were not significantly different for patients with or without a fast molecular response (supplementary Fig. 1).

3.5. *Outcome according to MRD status after completing induction chemotherapy*

After completion of induction chemotherapy, MRD was still detected in BM of 12 of the 29 (41%) patients. All patients with MRD positive BM died of progressive disease, while the 5-y-OS for patients with MRD negative BM was 52.3 ± 12.3%, Fig. 3c (log rank test, $p < 0.001$). Partial response, presence of residual ^{123}I-MIBG positive metastasis, positive BM cytology and the presence of urinary metabolites were also univariate significantly associated with poor survival. These results indicate that, residual disease present (especially in the BM) after the completion of induction therapy is indicative for resistant or progressive disease. qPCR remained significantly associated with survival when corrected for the other prognostic factors in separate (bivariate) models (Table 4).These results suggest that qPCR after completing of induction therapy can give additional information to currently used clinical parameters for assessment of disease status. For example, cytology detected NB cells in five BM samples, while qPCR detected 12 positive samples. Furthermore, qPCR identified 3 extra patients with residual disease, who were in CR according to INRC. The other patients with MRD positive BM never reached VGPR and had progressive disease before autologous harvest and/or stem cell reinfusion could be performed. Again at this time point a panel of markers identified more positive samples than individual markers. As an illustration, TH detected MRD in 6, PHOX2B in 11 and the panel of markers in 12 BM samples.

3.6. *Sequential MRD analysis*

BM samples of both follow-up time points (mid induction and after completion of induction chemotherapy) were available of 19 patients. Six patients were MRD negative at both time points (fast responders), 7 patients were positive at the first time point and negative after completing induction chemotherapy (slow responders) and 6 patients were still MRD positive after induction chemotherapy (non-responders). Although this cohort was very small, the survival curve indicates that patients in the first group with a fast molecular BM response had a better survival than patients in the other two

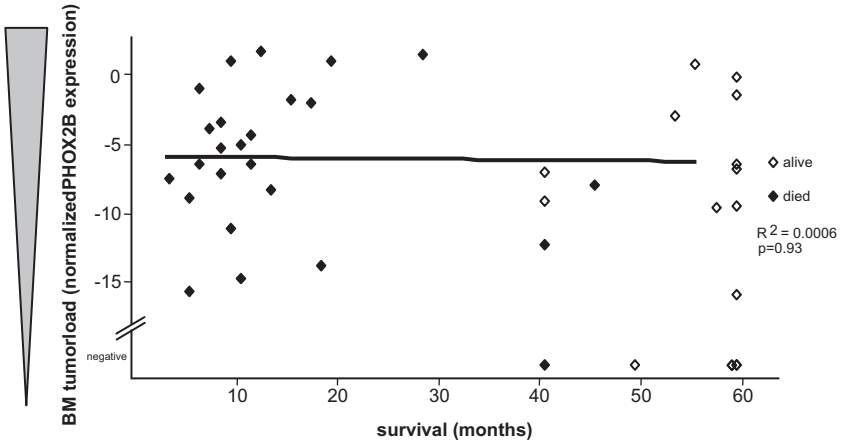

Fig. 2 – Correlation between tumour load at diagnosis and survival. Normalised expression of PHOX2B is shown in correlation with months survival, a cut-off at 60 months after diagnosis was set (Spearman's test, $p = 0.93$).

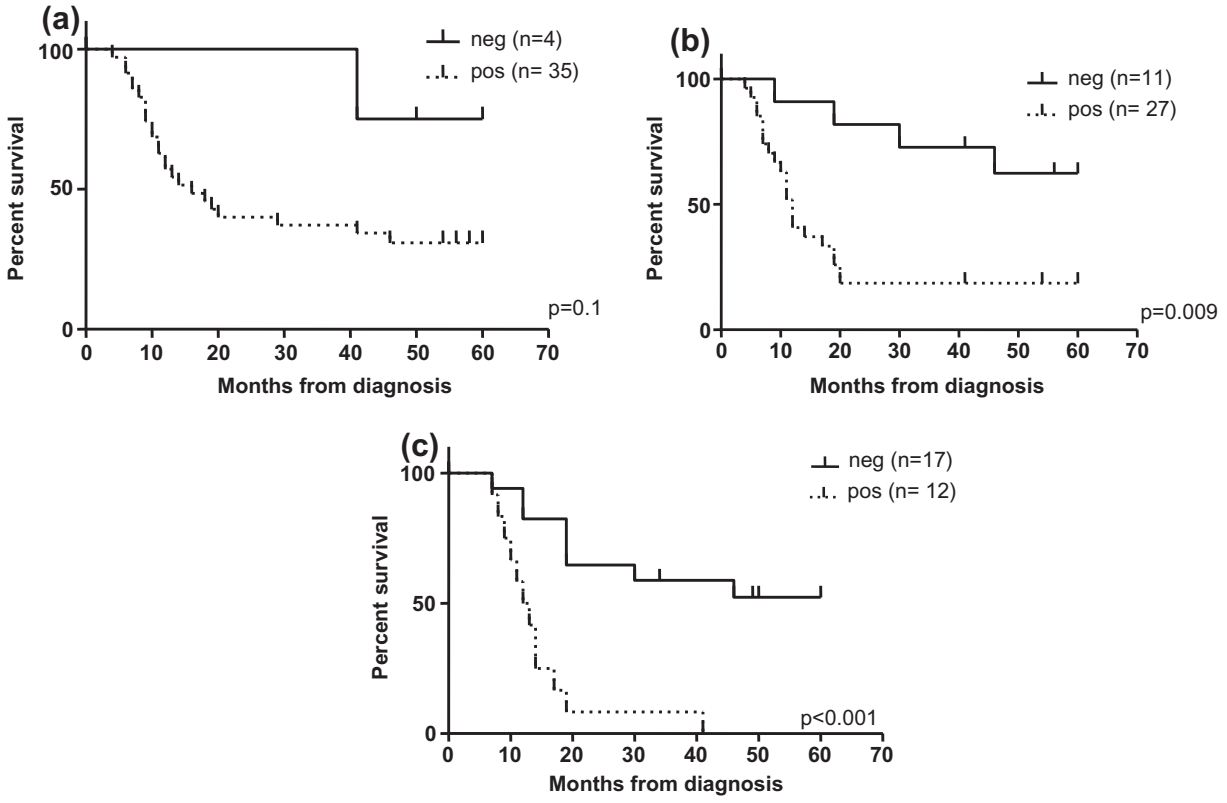

Fig. 3 – MRD detection at different time points during treatment is correlated with outcome. Survival curves according to MRD status at 3 months after diagnosis (a) at 3 months after diagnosis (b) and after completion of induction chemotherapy (c) (dotted curve, MRD positive patient; black curve, MRD negative patients).

groups (5-y-OS 62.5 ± 21.3%, 28.6 ± 17.1% and 0%, respectively, log rank test $p = 0.001$) (Fig. 4).

The levels of marker expression at all three time points were available from 14 of 19 patients. Representative examples of three patients, a fast responder, a slow responder and a non-responder are shown in Fig. 5 (for all 19 patients see supplementary Fig. 2). Although there were differences in MRD positivity between markers, the variation between the markers was not extensive and the changes with time were similar for the five PCR targets.

4. Discussion

In this study we have shown that sequential molecular MRD assessment of bone marrow is a prognostic factor for ultimate overall survival. We investigated this by measuring molecular bone marrow infiltration at (1) diagnosis, (2) early during treatment and (3) after completion of induction therapy. We found that a fast molecular response (BM negative by qPCR at mid induction) was associated with better outcome, compared to slow molecular response (BM negative

Table 3 – Measurements of response to treatment: Univariate and bivariate Cox regression analyses of survival factors, for all patients tested by qPCR at 3 months after start of induction therapy ($n = 38$).

Univariate analysis	p-Value	Hazard ratio	95% CI of the hazard ratio
qPCR (positive versus negative)	0.009	4.2	1.4–12.4
INRC response (CR/VGPR/PR versus MR/NR)	0.04	2.9	1.1–7.7
MIBG status (positive residual versus negative)	0.37	1.46	0.6–3.3
Catecholamine metabolites (CR/VGPR versus PR/MR/NR)	0.26	1.9	0.6–5.5
Cytology (positive versus negative)	0.12	2.0	0.8–4.6
Bivariate analysis[a]			
qPCR (positive versus negative)	0.05	3.3	1.0–10.8
INRC response (CR/VGPR/PR versus MR/NR)	0.38	1.7	0.58–5.0

All parameters are compared at diagnosis versus after 3 months therapy.
Abbreviations: INRC, International Neuroblastoma Response Criteria; CR, complete remission; VGPR, very good partial response; MR, mixed response; NR, no response.
[a] Only factors significant in univariate analyses were assessed in bivariate analyses.

Table 4 – Measurements of response to treatment: Univariate and bivariate Cox regression analyses of survival factors, for all patients tested by qPCR after completion of induction therapy (*n* = 29)

Univariate analysis	*p*-Value	Hazard ratio	95% CI of the hazard ratio
qPCR (positive versus negative)	<0.001	6.0	2.2–16.2
INRC response (CR/VGPR versus PR/MR/NR)	0.03	2.7	1.1–6.8
MIBG status (positive residual versus negative)	0.04	2.6	1.1–6.6
Catecholamines metabolates (CR versus VGPR/PR/MR/NR)	0.001	5.8	2.0–17.3
Cytology (positive versus negative)	<0.001	14.4	3.6–57.6
Bivariate analysis[a]			
qPCR (positive versus negative)	0.003	5.1	1.8–14.8
INRC response (CR/VGPR versus PR/MR/NR)	0.35	1.6	0.6–4.4
qPCR (positive versus negative)	0.003	4.8	1.7–13.6
MIBG status (positive residual versus negative)	0.25	1.8	0.7–4.8
qPCR (positive versus negative)	0.05	3.3	1.0–11.3
Catecholamine metabolites (CR versus VGPR/PR/MR/NR)	0.11	2.7	0.8–9.7
qPCR (positive versus negative)	0.007	4.5	1.5–13.4
Cytology (positive versus negative)	0.01	6.5	1.5–27.2

All parameters are compared at diagnosis versus after completion of induction therapy.

Abbreviations: INRC, International Neuroblastoma Response Criteria; CR, complete remission; VGPR, very good partial response; MR, mixed response; NR, no response.

[a] Only factors significant in univariate analyses were assessed in bivariate analyses.

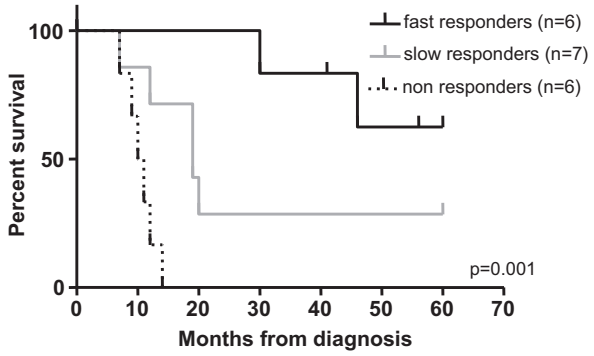

Fig. 4 – Sequential MRD analysis identifies fast responders. Fast responders were defined as MRD negative at 3 months after start of therapy and MRD negative after induction chemotherapy; slow responders were defined as MRD positive at 3 months after start of therapy and MRD negative after induction completion of chemotherapy; no molecular remission was defined as MRD positive 3 months after start of therapy and MRD positive after completion of induction chemotherapy (blackcurve, fast responders; grey curve, slow responders; dotted curve, no molecular BM remission during treatment).

by qPCR post induction) or no molecular remission after induction therapy.

The results can be of great translational value, even though the patient cohort described was small and not uniformly treated nor all treated according to treatment protocols employed today. In the light of our results, it might be possible to stratify patients based on MRD results already in the first months after diagnosis, independent of the treatment protocol. Also in acute lymphoblastic leukaemia it has been shown that an early molecular response is prognostic for outcome, regardless of the treatment protocol used.[14] Of course our results need to be confirmed in prospective MRD studies with uniformly treated patients. However, at this moment early time points are not included in most prospective MRD studies, as recommended by the International Neuroblastoma Risk Group Task Force.[15]

A number of authors have applied MRD detection by qPCR in PB or BM to assess the effect of induction chemotherapy,[7,8,16] adjuvant therapy[6] or total treatment[16–18] and to detect relapse.[8,19] The most commonly used markers in these studies were *TH* and *GD2 synthase* (*GD2S*). However, the use of *TH* and *GD2S* as MRD markers in neuroblastoma is hampered by their expression in normal BM, PB and/or PBSC. Previously, we have identified the marker *PHOX2B*, which is exquisitely neuroblastoma specific using the primer-probe set described[2] although using a different primer-probe set a single amplification in 1 out of 50 control PB/BM samples has been encountered by Viprey et al.[20] In addition, we described that a panel of markers is more sensitive for detection of MRD than the use of *PHOX2B* alone.[9] *GD2S* was not included in that panel, because of lower specificity and sensitivity than the other markers. Here we show that MRD detection in bone marrow with this panel of five qPCR markers is both clinically relevant and significantly associated with outcome. When we performed survival analysis per individual marker, *PHOX2B* was most significant for predicting outcome at both time points. Although the increase in detection of positive samples using a panel of five markers was minimal, TH and CHRNA3 identified 2 more patients with MRD positive BM that both died of progressive disease. On the one hand this further corroborates the superiority of applying a panel of markers compared to a single marker. However, on the other hand, the costs and use of the amount of sample are higher when using a panel of markers. So, our ongoing prospective study will show which markers will be needed for optimal MRD detection. Therefore, we advise to use next to PHOX2B at least one other marker, e.g. *TH* for MRD detection.

For the evaluation of the clinical relevance of MRD detection, we first determined whether the level of bone marrow

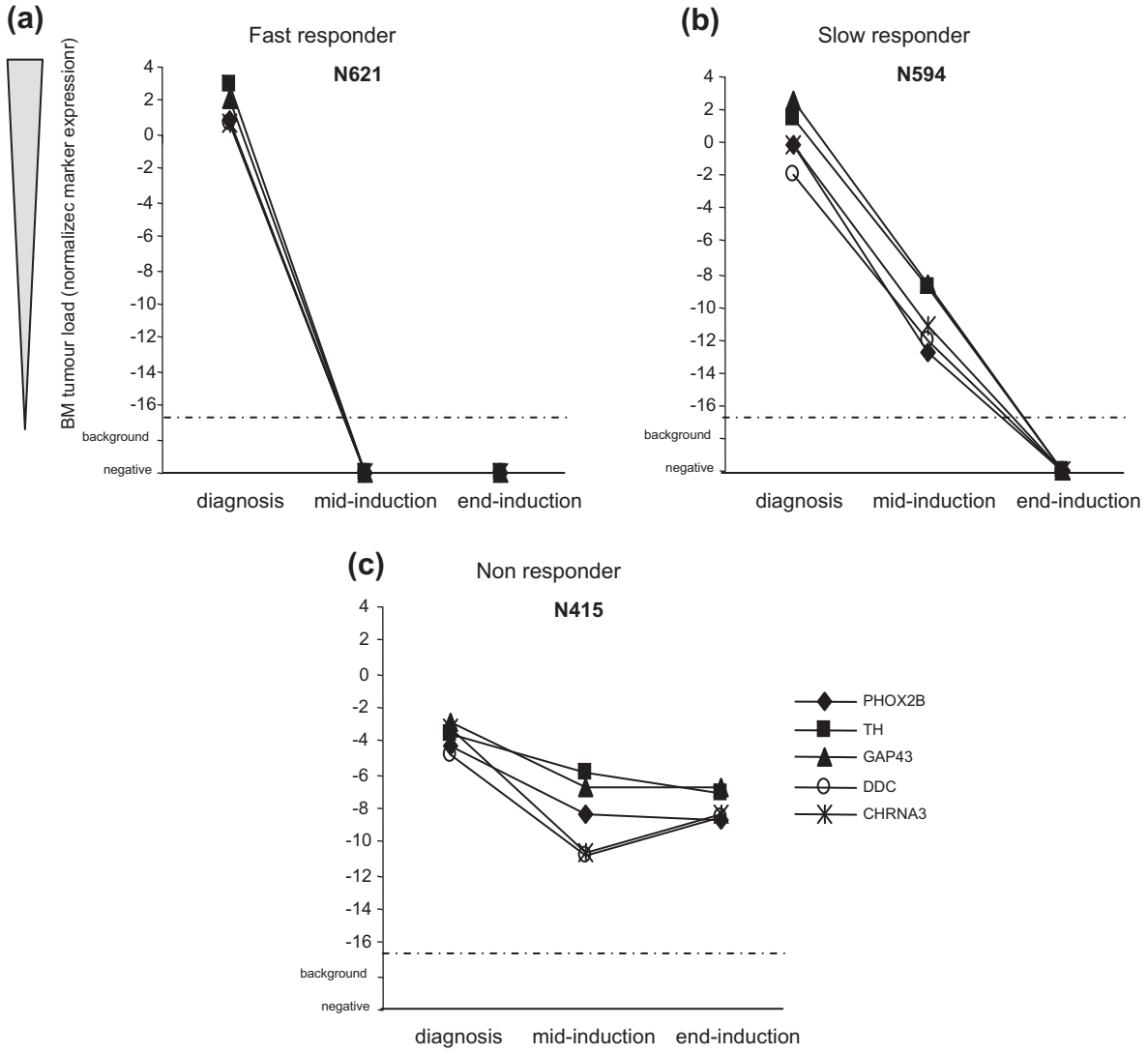

Fig. 5 – Normalised expression of 5 PCR markers at diagnosis, mid induction and end induction. Representative examples of a fast responder (a), slow responder (b) and a non-responder (c) are shown. *Abbreviations:* BM, bone marrow; background, normalised expression equal to control bone marrow samples. Patients are anonymised using *N* + patient number.

infiltration at diagnosis related to outcome. Remarkably, we did not find a difference in BM tumour load between patients who succumbed or survived. It has been suggested by Trager and colleagues[21] that high expression of *TH* at diagnosis corresponds to poor outcome. However, in their study all high risk neuroblastoma patients, including patients without metastasis with MYCN amplification, were selected, whereas we only tested BM of patients with stage 4 with bone or BM disease at diagnosis. In addition, different cut-off levels for defining MRD positivity were used, making it difficult to compare results between our study and the study of Trager and colleagues However, in both studies it seems that high tumour load at diagnosis is not correlated to survival. This suggests that the mere presence of BM infiltration is the major prognostic factor rather than the level of infiltration.

Secondly, we investigated whether the kinetics of molecular BM response to chemotherapy related to outcome. We first looked at the MRD status at 3 months after diagnosis and found that the absence of MRD was significantly associated with overall survival and remained significantly associated when tested in a bivariate model with INRC response. Only a few other studies investigated the impact of early clearance of BM MRD on outcome.[5,7,8] Using immunocytology, Seegers and colleagues[5] found that patients with more than 100 cells/10^5 in the BM after three cycles of induction chemotherapy had poor survival. However, in their study the number of MRD positive patients was only 6% compared to 71% in this study, because the level of detection was one tumour cell in 10^3 normal cells. In our study the level of detection for MRD positivity is one tumour cell in 10^6 normal cells.[9] Consequently, they identified patients resistant to therapy rather than the fast responders that we identified. Using *TH* as a marker, two small cohort studies also showed in univariate analysis that MRD detection with qPCR at 3–4 months after diagnosis was associated with poor outcomes.[7,8] Fukuda and colleagues[7] found that 6/21 (29%) patients had *TH* negative BM 4 months after diagnosis and none of these patients died. In the second study, Tchirkov and colleagues[8] found that

11/22 patients, with less than 1000 *TH* transcripts in the BM after three cycles of induction chemotherapy had a significantly better survival. However, both studies did not investigate the association of PCR results with other clinical response parameters.

In addition to the early time point, we also tested molecular response after completion of induction chemotherapy, and found that the presence of BM MRD was associated with poor outcome. In fact all patients whose BM was MRD positive after completion of induction therapy, ultimately died from their disease. We could not perform complete bivariate analysis which included all prognostic variables, because of the small sample size and performed, therefore, combined (bivariate) analysis only. In the combined analysis with INRC response, the presence of MRD after completion of induction chemotherapy remained significantly associated with survival. Three of 12 MRD positive patients were thought to be in CR, which strongly suggests that minimal residual disease can contribute to disease progression. Hence, our study shows that BM qPCR can give information, which cannot be obtained with the currently used clinical parameters for assessment of disease status such as INRC response.

In 20 out of 53 patients BM/PBSCs were not infused, because of the progression of disease. As can be expected none of these patients were fast responders, and of those analysed after induction chemotherapy one was a slow responder and all the others were non-responders. We are aware that most of these patients were treated with low intense chemotherapy and dose intense induction chemotherapy, which is now being used,[22] will probably result in more fast responders, which is currently being investigated in a prospective study. Our patient cohort did not receive adjuvant immunotherapy, so possibly more slow or non responders could have become MRD negative as described by Cheung and colleagues.[6,16] They showed with *GD2S* qPCR that patients with MRD positive BM before immunotherapy, who became MRD negative after two cycles of immunotherapy had a significantly better outcome than non-responders to immunotherapy. In addition they showed by using *PHOX2B* for MRD detection that almost all patients with BM positive disease after chemotherapy and two cycles of immunotherapy died of progressive disease,[23] which is in line with our results.

In only a limited number of patients ($n = 19$) MRD could be assessed at the two consecutive time points. Despite the small size of this study and the variation in treatment protocols, our results indicate that, based on the kinetics in treatment response, three patient groups (within the high risk group) can be identified with different prognosis (Fig. 4); an extremely high risk group with no molecular BM remission, a very high risk group with slow molecular BM response (molecular remission post induction) and a high risk group with a fast molecular BM response already in molecular remission after 3 months of therapy. In addition, a very small group has stage 4 disease without measurable BM invasion, which also might have a better response to HR treatment.

We conclude that MRD detection as measured by a panel of NB specific-PCR targets, constituted by PHOX2B, TH and CHRNA3, can identify fast responders, who clear their BM early during treatment. Early molecular response seems to be a prognostic factor associated with better outcomes. Our results indicate that it might be possible to stratify patients based on MRD results already in the first months after diagnosis. PCR guided therapy might result in better survival rates by early identification of patients who might benefit from conventional treatment and early identification of patients who need different (new) therapies. To verify the results of this small heterogeneous retrospective study, we strongly recommend including time points during induction therapy in prospective MRD studies.

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Conflict of interest statement

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2011.02.003.

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